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Virulence of Pigmented *Serratia marcescens* Strain SM6 and its Nalidixic Acid-Resistant Derivative in White Outbred Mice

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Abstract Human opportunistic pathogen *Serratia marcescens* is a bacterium with broad host range representing a growing problem for public health. Little is known about virulence factors used by *S. marcescens* to colonize the host. We used white outbred mice to study virulence of *S. marcescens* wild type strain SM6 and its nalidixic acid-resistant derivative during intraperitoneal infection. We showed that both strains were lethal for mice at 10^7–10^8 CFU and death occurred within 24–48 h post infection. No mortality was noticed at 10^6 CFU. Bacteria were isolated from hearts, lungs, kidneys, livers, spleens, intestines, and blood of infected animals. Resistance to nalidixic acid did not affect virulence of *S. marcescens*. Thus, this strain can be used in the future virulence studies to simplify tracking and recovery of *S. marcescens* from infected tissue.

Keywords *Serratia marcescens* · White outbred mice · Virulence

1 Introduction

Gram-negative bacterium *Serratia marcescens* can cause sepsis, endocarditis, and infections of respiratory and urinary tract. This microorganism recently started to draw more attention due to emergence of strains with resistance to multiple antibiotics [1]. Nevertheless, our knowledge about virulence of *S. marcescens* is limited in part due to the lack of established animal models. Known *S. marcescens* virulence factors include proteases, nucleases, lipopolysaccharides, iron uptake system, and hemolytic enzymes [2, 3]. To expand the list of virulence factors employed by *S. marcescens*, it is crucial to recover bacteria from the infected tissue to directly compare the impact of individual genes on the infection outcome. That could be achieved through the use of fully virulent strain with antibiotic resistance.

Spontaneous resistance to streptomycin and rifampicin could easily arise; however, in *Salmonella* Typhimurium those mutants are avirulent in mice [4]. Animal experiments with well-known pathogen, *Salmonella* Typhimurium, showed that spontaneous mutation in *gyrB* gene [5] resulted in nalidixic acid resistance that did not affect virulence in mice and thus, provided a strategy for many research groups to recover bacteria from the infected tissue [6–8]. In contrast, spontaneous mutations in *gyrA* gene of *Salmonella* Typhimurium were reported to cause attenuation of virulence in some cases [4]. The impact of nalidixic acid resistance of *S. marcescens* on virulence was not previously investigated. The aim of this study was to evaluate the virulence of pigmented *S. marcescens* strain SM6 and its nalidixic acid-resistant derivative in intraperitoneal infection of white outbred mice.

2 Materials and Methods

Serratia marcescens strain SM6 (LMB1) was obtained from Dr. Michael Benedik (Texas A&M University, USA). Strain LMB40 is a spontaneous nalidixic acid-resistant derivative of SM6. Both strains were routinely grown on LB media. Nalidixic acid was used at 50 mg/l when appropriate.

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Bacterial cultures were grown overnight at 37 °C with shaking (250 rpm). Bacterial cells were collected by centrifugation (max speed for 5 min) and washed once with sterile 0.9 % NaCl. Resulting bacterial cells were resuspended in 1×PBS and used for infections.

For evaluation of hemolytic and proteolytic activity, SM6 and LMB40 strain was gown overnight at 37 °C with shaking (250 rpm). Cells were collected by centrifugation as described above, washed twice in 1×PBS, and resuspended in 500 μL of phosphate buffer. Five microliters from bacterial suspension of each strain (OD600 = 0.5) were spotted onto the center of blood or skim milk agar plates. Hemolytic activity was tested on Columbia agar with 5 % sheep blood (Bio-Rad). Proteolytic activity was tested on skim milk agar plates containing 1 % tryptone, 0.5 % yeast extract, 1 % NaCl, and 3 % powdered skim milk. Plates were incubated for 3 days at 37 °C.

Methanol and acetonitrile fractions of *S. marcescens* SM6 conditioned media were analyzed using high-resolution LC-QTOF maXis II (Bruker, Germany) combined with HPLC UltiMate® 3000 Nano LC systems (Thermoscientific). MS instrument was operated in positive ESI mode. HPLC was equipped with thermostatted autosampler. Column Acclaim PepMap RSLC, nanoViper, C18, 2 μm, 100 Å, 75 μm × 15 cm. The temperature of the column oven was 40 °C. The mobile phase consisted of water/acetonitrile/formic acid (A = 94.9 % water/5 % acetonitrile/0.1 % formic acid; B = 94.9 % acetonitrile/5 % water/0.1 % formic acid) at flow rate 0.4 mL/min. The mobile phase gradient was linearly increased from 0 to 2 % (vol/vol) during 5 min, 2 to 50 % (vol/vol) during 15 min, 50 to 90 % (vol/vol) during 1 min, and then 90 % of mobile phase B was maintained for 7 min with followed decreasing to 2 % (vol/vol) of acetonitrile for 2 min and to the next injection. Volume of injected sample was 10 μl.

White outbred mice were used at 12 weeks of age (with average body weight of 18 g). All experiments were approved by Institutional Animal Care and Use Committee of Kazan Federal Center for Toxicological, Radiation, and Biological Safety.

For virulence studies, mice were divided into three groups (five animals per group) and intraperitoneally infected with 10^6, 10^7, and 10^8 of either SM6 or LMB40 in 0.5 ml, respectively. Control group was injected with the equal volume of 1×PBS. Animals were monitored for 14 days post infection. In additional experiments, three animals from group infected with 10^6 bacteria were sacrificed on days 1 and 7. Lungs, livers, spleens, intestines, kidneys, hearts, and blood were collected for isolation of *S. marcescens*. Resulting colonies were confirmed using MALDI Biotyper (Bruker) according to manufacturer's recommendations. Scores were calculated by Biotyper software as arbitrary units with values between 0 and 3 as a result of comparison of each sample mass spectrum to the reference mass spectra in the Bruker database. Scores below 1.7 were considered unreliable. Scores ≥ 2 were accepted for species assignment.

3 Results and Discussion

We showed that both *S. marcescens* strains, SM6 and LMB40, were capable of secreting comparable amount of hemolytic and proteolytic enzymes during growth on blood and on skim milk agar plates, respectively (Fig. 1). Moreover, both hemolysin and serralysin were detected in the conditioned media of *S. marcescens* SM6 by mass-spectrometry (MaXis UHR-TOF) (data not shown).

Intraperitoneal infection of white outbred mice with 10^6 SM6 or LMB40 led to temporal reduction in animal activity in both experimental groups on day 1 post infection. Starting from day 2, animals resumed normal activity; no mortality was noticed throughout the duration of experiment. At 10^7 infectious dose, approximately 80 % of animals in both groups died after 24 h post infection, no survivors remained after 48 h post infection. Animals infected with 10^8 SM6 or LMB40 succumbed to infection within 24 h post injection (Fig. 2).

Analysis of changes associated with 10^6 intraperitoneal infections of both SM6 and LMB40 on days 1 and 7 post infection showed that mesentery, subcutaneous adipose tissue, and the peritoneal walls of infected animals were hemorrhagic, blood vessels were dilated. Intestinal lumen was filled with yellow mucus. *S. marcescens* was isolated from heart, lung, kidney, liver, spleen, intestines, and blood of infected animals on both days. Isolated cultures were confirmed as *S. marcescens* by MALDI Biotyper with score values 2.173–2.292. No significant changes were observed in the spread of nalidixic acid-resistant strain LMB40 compare to its parental strain SM6.

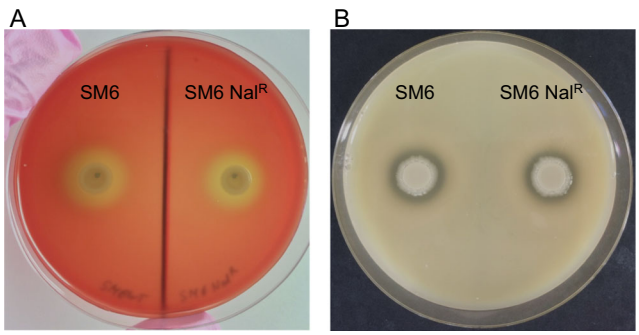

Fig. 1 *Serratia marcescens* SM6 and its nalidixic acid resistant derivative strain secrete similar levels of hemolytic (**a**) and proteolytic (**b**) enzymes on blood and skim milk agar plates, respectively. Pictures were taken after 3 days of incubation at 37 °C

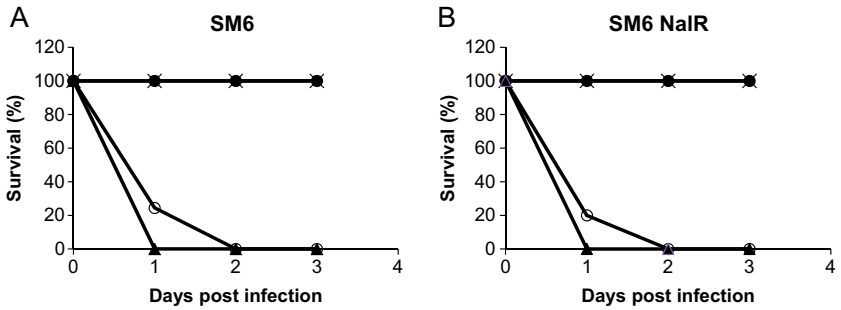

Fig. 2 *Serratia marcescens* SM6 and its nalidixic acid resistant derivative are virulent in white outbred mice. Twelve-weeks-old mice were intraperitoneally infected with **a** *S. marcescens* SM6 or **b** *S. marcescens* LMB40 with 10^6 (*filled circles*); 10^7 (*open circles*); 10^8 (*triangles*) bacteria or injected with an equal volume of PBS (*cross*)

4 Conclusion

Our study showed that pigmented strain SM6 is virulent in white outbred mice and can spread and persist in all organs tested for at least 7 days post infection. Spontaneous mutation that resulted in resistance to nalidixic acid did not affect virulence of *S. marcescens* and thus provides a tool for the future virulence studies using antibiotic selection as a way to track and recover *S. marcescens* from infected tissue.

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